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THE TOXINS AND ANTITOXINS OF POISONOUS MUSHROOMS (*AMANITA PHALLOIDES*).*

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1. HISTORICAL INTRODUCTION.

The deaths annually from consumption of poisonous fungi, generally known as toadstools, are so numerous, especially in countries where fungi are an important and valuable constituent of the food of the poorer classes, while therapeutic measures are so notoriously use-

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less in the treatment of the severe cases, that investigations into their toxicological properties always have practical importance. The actual mortality from this cause is difficult to estimate. There are, however, a number of notable individuals in early times who lost their lives from eating poisonous mushrooms, among whom may be mentioned the daughter of the historian Pausanias, the emperor Claudius, King Charles VI of France, the pope Clement VII, and the Princess of Conti, while in modern times the most prominent example is the Czar Alexis of Russia. For over a century fatal cases have been reported in medical periodicals, and during the latter part of the 18th century Paulet[1] found 100 deaths from this cause in the vicinity of Paris. Later Bardy[2] collected 60 cases in the Vosges, covering a period of six years. In 1885 Guillaud[3] estimated the annual number of deaths in the southwest of France at about 100 cases.

In Japan fatalities from fungus poisoning are numerous, Inoko[4] having reported 481 cases in a period of eight years. In other countries they are frequent, for Falck[5] has collected 53 in Germany with 40 deaths, and there are numerous reports of individual groups in the German literature.

In America, Palmer,[6] of Boston, has found 33 cases with 21 deaths, and Forster,[7] of Charlestown, 44 cases with 14 fatalities.

Such statistics do not represent adequately the real loss of life, since the majority of cases do not find their way into medical literature. Thus during the past summer there were two cases in Baltimore, fortunately neither fatal; a family was poisoned in Cleveland, Ohio, with two deaths; nine persons in Fostoria, Ohio, with several deaths; ten individuals in Toronto, with two deaths; and Pfromm[8] of Philadelphia has just reported the poisoning of four members of an Italian family with four deaths. In addition Plowright[9] has recently reviewed one of his earlier cases to which he has added three other deaths in England.

The three most important varieties of poisonous fungi are *Amanita muscaria*, *Helvella esculenta*, and *Amanita phalloides*. From the first of these, *Amanita muscaria*, known as the "Fly Amanita," from its extensive use as a fly poison, Schmiedeberg[10] and his pupils, Koppe and Harnack,[11] have extracted the alkaloid muscarin, which they have shown by extensive animal experimentation to be responsible for the symptoms and lesions found in cases of muscarin poisoning in man. Later muscarin was prepared synthetically by the oxidation of cholin, and its chemical constitution has been estimated as $C_5H_{15}AZO_3$. Artificial muscarin differs materially from the natural product, in its action upon animals. The *Amanita muscaria* is employed in large quantities by the peasants of the Caucasus, who prepare an intoxicating beverage, from the excessive use of which many individuals die annually. Poisoning from eating *Amanita muscaria* is comparatively rare, since this fungus has a bitter, unpleasant taste, and in consequence is not eaten in any great quantity. The cases seldom terminate fatally, and the symptoms are referable to the profound action of muscarin upon the nerve centers. It has been shown experimentally that atropin is a perfect physiological antidote for muscarin, and the use of this drug has frequently been found of great value in severe cases of poisoning. (See the report of Prentiss[12] concerning the poisoning of the Count de Vecchi and his physician, Dr. K., the latter of whom recovered from muscarin poisoning after treatment with excessive doses of atropin.)

The second poisonous fungus, *Helvella esculenta*, owes its toxicity to helvellic acid, a substance isolated by Boehm and Külz,[13] who give it the structural formula $C_{12}H_{20}O_7$, and who have shown that it is strongly hemolytic in its properties. Numerous fatalities among the German peasants were formerly attributed to this fungus, but very few deaths have been reported within recent years for which *Helvella esculenta* could be held definitely responsible.

The most important of all the poisonous fungi, because of its great abundance, its superficial resemblance to the edible mushroom, its innocent appearance, its delicious taste, and, finally, its extreme toxicity is the *Amanita phalloides*, the small or spring form of which is called the *Amanita verna*.

This is a pure white fungus growing in damp woods, and occasionally appearing in open sunny fields which abound in meadow mushrooms, with which it is rarely mixed. Its botanical characters are well marked. It varies in height from four to six inches, with a top or pileus about two and one-half inches in diameter. The gills and the attached spores are white. The stalk is white and slightly fibrous. About the upper part of the stalk is a definite veil, and the base of the stalk is surrounded by a large cup or volva sunk deep beneath the surface of the ground. This cup is known as the "death cup," and its bulbous shape has given the fungus its French name, *Amanita bulbeux.*

The pileus is usually pure white, but forms have been described where the pileus is colored green, yellow, or brown. The white forms are by far the most common, at least in this country.

The small form, the *Amanita verna*, grows to a height of two or three inches, with a pileus not more than one inch in diameter. The toxic properties of the *Amanita verna* have been so well recognized in France that Bulliard,[14] the most eminent of French mycologists, nearly a hundred years ago gave it the name "destroying angel." In England and in America the *Amanita phalloides* is known as the "Deadly Amanita."

The deadly amanita is of extreme toxicity. Plowright[15] has reported the death of a child of 12 years from eating a third of the top of a small plant, while there are many cases on record where adults have perished from eating two or three good-sized specimens. Indeed, a long study of the literature of fungus poisoning leaves little room for doubt that practically all the deaths from mushrooms are to be attributed to this one species.

The deaths in France from the consumption of *Amanita phalloides*, were collected in 1900 by Gillot,[16] who reports 123 fatalities in which the fungus eaten was definitely identified. In addition to Gillot's cases I have been able to find in the German, Italian, and English literature, and in the French since 1900, nearly 200 deaths from the same fungus, so that there are a sufficient number of cases reported in detail to give us accurate knowledge of their symptoms during life and of their postmortem lesions.

PATHOLOGICAL CHANGES.

The earliest careful study of the pathological changes found in phalloides poisoning was made by Maschka,[17] who summarized these changes as:

1. Lack of postmortem rigidity;
2. Widening of the pupils;

3. Failure of the blood to coagulate, and a cherry-red color;

4. Ecchymoses and hemorrhages in the serous membranes and parenchymatous organs;

5. Dilatation of the bladder with urine.

Later Studer Sahli and Schärer,[18] of Bern, contributed a valuable series of papers to the subject of phalloides poisoning, treating it from the botanical, clinical, and pathological standpoints. They confirmed all of Maschka's observations, calling attention, to the extensive necrotic and fatty changes in liver, kidney, heart, and voluntary muscles. These lesions Maschka had seen in three of his seven cases, but had not considered essential.

The fatty degeneration of the organs and tissues is pronounced, and the amount of fat in the liver is extremely great, almost as abundant as in phosphorous poisoning. Thus Tappeiner[19] has estimated the fat content of the liver in two cases of *Amanita phalloides* poisoning as 53.6 per cent and 68.9 per cent as compared with a fat content in cases of phosphorous poisoning of from 50 to 70 per cent. The widest variation in the fat of normal livers is from 8.9 to 25.5 per cent (Perls). The most valuable confirmation of the work of Maschka, Studer Sahli and Schärer and Tappeiner has been that of Moers,[20] who examined the internal organs of three individuals dead of *Amanita phalloides* poisoning, testing them for arsenic, phosphorus, and muscarin. All of these substances were absent, so that any doubt as to the relationship between the fungus and the postmortem lesions was dispelled. In Moers's cases the microscopic changes present were identical with those described by the earlier observers.

CLINICAL SYMPTOMS.

The clinical symptoms following the ingestion of *Amanita phalloides* are characteristic. After a latent period of from six to twelve hours, during which the victims remain quite well, they are suddenly seized with terrible abdominal pain, with excessive vomiting and thirst. Diarrhea may set in with mucous, bloody stools, or the bowels may be constipated. Anuria is usually present. The paroxysms of pain may be so severe as to result in a peculiar Hippocratic facies, emphasized by the French as "la face vulteuse."

Periodical attacks of pain and vomiting alternate with periods when the symptoms seem ameliorated. The patients rapidly lose strength and frequently become jaundiced. After three to four days in children, and six to eight days in adults, coma develops, from which the patients cannot be aroused by any artificial stimulus, and which results in death within a few hours. Cyanosis and lowered temperature precede the fatal exit. Ocular symptoms and convulsions do not ordinarily occur, but convulsions may be present as a terminal event. The mortality in various epidemics is high, varying from 60 to 100 per cent, and depending largely upon the amount of the fungus eaten. Treatment is useless.

CHEMICAL INVESTIGATIONS.

The earlier chemical investigations upon *Amanita phalloides* were conducted by Boudier,[21] who isolated a substance in 1866 which he considered an alkaloid and which he named "bulbosin." A year later Latellier and Speneux[22] obtained a preparation which they named "amanitin," and which they believed to consist of an irritating and a narcotic principle. Finally Oré[23] extracted a substance called "phalloidin," which he later endeavored to prove was identical with strychnin.

It is impossible to come to any definite conclusion concerning bulbosin, amanitin, and phalloidin. None of these substances was obtained in a pure condition; the fungi employed for the investigations were mixtures of a number of different varieties, including *Amanita muscaria*, and it is generally believed by mycologists that the products mentioned were impure preparations of muscarin or cholin.

At all events, Kobert's[24] investigations in 1891 cleared up a great deal of the obscurity surrounding the matter. He collected a large quantity of fungi which were accurately identified as *Amanita phalloides*. From these fungi he obtained a substance characterized by its marked hemolytic action, his dried extract dissolving ox blood in a dilution of 1 : 125,000. To this hemolytic principle, extracted from *Amanita phalloides*, Kobert gave the name "phallin," and from its thermolability he considered it a toxalbumen. By intravenous injection with phallin Kobert produced intracorporeal laking of the blood, and attributed its toxic action to its hemolytic properties alone.

Since Kobert's investigations little of value has appeared in the literature.

Seibert,[25] working in Kunkel's laboratory, was unable to confirm Kobert's conclusions, but the value of Seibert's work is destroyed by the fact that he used a yellow fungus, *Amanita citrina*, which is seldom, if ever, toxic to man. His methods of extracting the fungus were not such as to bring out any hemolytic properties and he made no microscopic examinations of his experimental animals, so that we do not know whether the changes produced by other hemolytic agents, such as pigmentation of the spleen, were present or not.

As far as can be learned from a review of the literature no serious attempt has been made to study *Amanita phalloides* from the standpoint of serum-therapy, so in view of the great theoretical and practical interest which attaches to the subject of toxins and anti-toxins at the present time, it was determined to carry out a systematic investigation of poisonous properties with the object of determining whether antitoxins could be prepared for it. These investigations have now been pursued three years and it is the object of the present paper to report the most important of the results.

2. PREPARATION OF MATERIAL.

The fungi were collected in considerable quantity in the Blue Ridge Mountains of North Carolina, of Maryland, and of Pennsylvania, as well as in some of the thickly wooded regions in the vicinity of Baltimore. Only those specimens which corresponded to the standard botanical description of *Amanita phalloides* given by Atkinson,[26] Farlow,[27] Peck,[28] and others, were saved, and of these only the pure white forms have thus far been employed. The color of the pileus and its freedom from scales, the color of the spores, the fibrous character of the stem, the presence of a veil, and finally the presence of a poison cup at the base of the stem were considered essential characters in identification.

The fungi were dried either in direct sunlight or in especially prepared ovens, and the dried material was then preserved in the dark. A number of unsuccessful attempts were made to extract the fungi satisfactorily, during which much of the first year's collection was wasted. Finally a satisfactory method was elaborated and the same method has been followed throughout the investigations. The dried fungus is first carefully weighed and pulverized in a mortar. A definite quantity of water is now added and the material placed *on ice for 48 hours.* It is then expressed under considerable pressure, the juice thus obtained being dark brown in color with an odor similar to that of the fresh plant. At least three extractions are made from each lot of fungi. The extract is now filtered, first through fine filter paper, then through a Berkefeld filter. For hemolytic reactions concentrated NaCl solution is added in quantities sufficient to bring the salt content of the extract up to 1 per cent. The extract may be preserved almost indefinitely, but a small piece of thymol is usually added to each flask to prevent the development of molds.

As thus prepared, the extracts differ slightly from those made by Kobert. It being highly desirable to obtain all the constituents of the fungus and to preserve them intact, the extract was not evaporated to dryness lest some injury be done to any delicate poisons present. Evaporation, moreover, leaves a viscid, sticky mass which absorbs water so rapidly that it is impossible to measure exact quantities; so both for hemolytic work and for the inoculation of animals, the fluid extracts are preferable.

3. THE HEMOLYSIN PRESENT IN *AMANITA PHALLOIDES*.

The extract of *Amanita phalloides* is a powerful hemolytic agent. It quickly dissolves the erythrocytes of guinea-pig, rabbit, fowl, pigeon, dog, goat, and man. This reaction takes place rapidly at 37° C., somewhat more slowly at lower temperatures. The corpuscles are not agglutinated, and both washed and unwashed corpuscles are alike dissolved. The reaction takes place with considerable dilution of the crude extract as can be seen from Table 1.

The same solution of *Amanita phalloides* exerts practically the same action upon washed guinea-pig corpuscles. In the following table the corpuscles were washed several times in 0.75 per cent NaCl solution. The hemolysis is considerably more active with the washed corpuscles than with the unwashed, but the difference cannot be expressed in figures.

TABLE 1.

Lysis of Guinea-Pig Blood (Unwashed).

Amanita phalloides Extract I, prepared from 8 grams dried Amanita and 100 c.c. distilled water. Soaked 48 hours on ice, expressed under pressure, filtered through filter paper and then through Berkefeld filter. Concentrated NaCl solution added to make the solution 1 per cent NaCl.

Extract I +5 per cent Blood 1.0 c.c.	Lysis
Extract 1.0 c.c.	Complete
" 0.1	"
" 0.006	"
" 0.004	Almost complete
" 0.002	Partial
" 0.001	None
NaCl Sol. only (control)	"

Observations made at the end of one, two, and five hours. Final readings taken at the end of 18 hours. The same procedure followed in all hemolytic experiments.

The corpuscles are not agglutinated.

TABLE 2.

Lysis of Guinea-Pig Blood (Washed)

Extract I +5 per cent Blood 1.0 c.c.	Lysis
Extract 1.0 c.c.	Complete
" 0.1	"
" 0.01	"
" 0.006	"
" 0.004	Almost complete
" 0.002	Partial
" 0.001	None
NaCl Sol. only (control)	"

From these two tables it may be seen that complete solution of both washed and unwashed guinea-pig corpuscles occurs with this extract of *Amanita phalloides* in quantities representing 0.006 c.c. The same extract tested upon rabbit corpuscles is somewhat less powerful, dissolving these erythrocytes completely in a dilution of 1 to 100 or 0.01 c.c. The washed and the unwashed corpuscles are dissolved with practically the same quantities of the extract. Erythrocytes from various species of animals exhibit great differences in susceptibility to *Amanita phalloides* as they do to most hemolytic agents. Thus Extract I which dissolves guinea-pig blood in a dilution of 0.006 c.c. and rabbit blood in a dilution of 0.01 c.c. has much less action upon fowl, dog, and pigeon corpuscles which are completely dissolved by 0.06 c.c., 0.08 c.c., and 0.2 c.c., respectively. With dog blood an abundant, brown, flocculent precipitate is given by

Amanita phalloides extracts, and the appearance of this precipitate marks the limits of hemolysis. Dog serum does not give this reaction, but washed corpuscles, freed from serum and then laked with minimum quantities of water give it abundantly. It is apparently some reaction between the phalloides extract and the albuminous constituents of the dog erythrocytes, while at the same time some reducing action may be exerted upon the oxyhemoglobin. The same flocculent precipitate appears with pigeon blood, and here, too, this marks the limits of hemolysis. This precipitate also is given by laked pigeon corpuscles. When examined microscopically the nuclei of the pigeon corpuscles are always found intact.

The following table gives a synopsis of the hemolytic reactions of Extract I, and the relative susceptibility of various erythrocytes.

TABLE 3.

SYNOPSIS EXTRACT I.

Species	Quantity of Extract I Necessary to Cause Complete Solution of 1 c.c. of 5 per cent Blood Suspension
Guinea-pig	0.006 c.c.
Rabbit	0.01
Fowl	0.06
Dog	0.08
Pigeon	0.2

Other extracts of *Amanita phalloides* have been obtained which are considerably stronger than the one mentioned above. Thus an extract was obtained from 15 grams powdered *Amanita phalloides*, and 200 c.c. distilled water, which dissolved guinea-pig corpuscles in a dilution of 1:1000 and rabbit corpuscles in a dilution of 1:500. This extract was tested carefully upon human blood corpuscles which were found relatively susceptible, as can be seen in the following table, which gives the result obtained with washed and unwashed corpuscles:

TABLE 4.

LYSIS OF HUMAN BLOOD.

Extract IV +5 per cent Blood 1.0 c.c.	Lysis
Extract 1.0 c.c.	Complete
" 0.1	"
" 0.05	"
" 0.03	"
" 0.02	"
" 0.01	None
NaCl only (control)	"

Hemolytic strength for human blood 0.02 c.c.

This extract was also studied with a variety of other erythrocytes, including goat, sheep, swine, and beef. Goat blood was found quite resistant, 0.5 c.c. of the crude extract being necessary to dissolve completely 1 c.c. of a 5 per cent suspension. Swine, sheep, and beef blood were found insusceptible to this particular preparation.

The following table gives a synopsis of the reactions of this extract.

TABLE 5.

SYNOPSIS EXTRACT IV.

Species	Quantity of Extract IV Necessary to Dissolve Completely 1 c.c. of 5 per cent Suspension of Blood	
Guinea-pig	0.001 c.c.	
Rabbit	0.002	
Man	0.02	
Goat	0.5	
Swine	Negative	Some discoloration of corpuscles
Beef	"	" " "
Sheep	"	" " "

PROPERTIES OF THE HEMOLYSIN.

From these experiments it can be seen that the hemolytic ingredient of *Amanita phalloides*, corresponding to the phallin of Kobert acts upon a variety of erythrocytes in a characteristic manner. Certain corpuscles, like those of guinea-pigs and rabbits, are very susceptible to its action while others are relatively resistant. Important questions at once arise as to the nature of this hemolytic material. Does it belong to the class of hemolytic principles found in normal blood serum, or in the blood serum of animals treated with alien corpuscles, which have been shown by Bordet and Ehrlich and their followers to consist of two substances, one uniting with the corpuscles (amboceptor, immune body, substance sensibilatrice), and another bringing about their solution (complement, alexin)? Or does it correspond to the bacterial hemolysins whose action is apparently exerted directly upon the susceptible erythrocytes without the intermediation of any complemental substances? Finally, can one be dealing with a hemolytic principle similar to that found in cobra venom, which was shown by Flexner and Noguchi[29] to belong to the class of serum hemolysins in that its hemolytic activity is dependent upon the intra-action of two substances, amboceptor and complement, but in which the complement has been shown by Kyes[30] to consist of lecithin?

Primarily extracts of *Amanita phalloides* dissolve blood corpuscles of various species without the intermediation of blood serum. Thus if corpuscles be washed in physiological salt solution seven or eight times, these corpuscles are still dissolved, and even more readily than are the unwashed corpuscles. Again, if extracts be added to susceptible corpuscles at low temperatures, such as 3° C., hemolysis does not take place. At the end of two hours, if these corpuscles be separated from the supernatant fluid by centrifugation, it is found that this fluid is robbed of its hemolytic properties completely, while the corpuscles, if again suspended in saline solution and placed at 37° C., are dissolved. A number of experiments were carried out, all demonstrating this point. The following experiment is typical:

An extract of *Amanita phalloides* of which $\frac{1}{10}$ c.c. dissolves 1 c.c. of a 5 per cent rabbit-blood suspension in a few minutes was added to washed rabbit corpuscles in equal quantities and placed on ice. At the end of two hours the corpuscles were neither agglutinated nor dissolved. The mixtures of extract and corpuscles were then centrifugalized. The supernatant fluid was no longer hemolytic for rabbit blood, while the corpuscles again suspended in 1 per cent NaCl solution were at once dissolved when placed in the thermostat.

The hemolytic principle of *Amanita phalloides* therefore can unite directly with corpuscles at low temperatures, and can dissolve these corpuscles when freed from all traces of serum, placed at a favorable temperature. In other words, this hemolysin is a single substance acting directly upon susceptible erythrocytes.

Secondarily, the temperature at which the hemolytic activity of *Amanita phalloides* is inhibited is much above that necessary to inactivate serum hemolysins (58.5° C.), and also above that established by Neisser and Wechsberg[31] for the bacterial hemolysin produced by cultures of *Staph. pyogenes aureus* (56° C.), and for bacterial hemolysins in general. Thus it requires a temperature of at least 60° C. before the extracts lose in hemolytic activity, while this activity is not entirely suspended until the temperature reaches 65° C.

INACTIVATION OF HEMOLYSIN OF *AMANITA PHALLOIDES*.

This is shown in the following table:

TABLE 6.

CONTROL.

EXTRACT IV+5 PER CENT BLOOD 1.0 C.C.	LYSIS			
	Unheated Extract	Extract heated to 58.5° C. 30 m.	Extract heated to 60° C. 30 m.	Exlract heated to 65° C. 30 m.
Extract 1.00 c.c.	Complete	Complete	Complete	None
“ 0.1	“	“	Partial	“
“ 0.05	“	“	None	....
“ 0.01	“	“	“	None
“ 0.005	“	“	“	....
“ 0.002	“	“	“	....
“ 0.001	Trace	Trace	“	None
NaCl sol. only (control)	None	None	“	“ “

From this table it may be seen that the hemolytic action of *Amanita phalloides* is partially suspended at a temperature of 60° C., but is only completely inhibited at 65° C. Similar experiments were carried out with a great variety of erythrocytes, and, as a general rule, a temperature of 65° C. acting for one-half hour suffices to inactivate this hemolysin for all kinds of blood. Occasionally certain strong extracts must be heated to 70° C. before they cease to exert a trace of hemolytic activity for such very susceptible erythrocytes as those of the guinea-pig.

ATTEMPTED REACTIVATION.

The attempt was made with these inactivated extracts of *Amanita phalloides* to find some substance which would restore to them their hemolytic properties. For this purpose a variety of blood sera, namely, that from guinea-pig, rabbit, fowl, swine, sheep, and beef, as well as cow's milk, were employed. The unheated *Amanita phalloides* was first tested upon rabbit blood to be certain that its activity was still present. It was then heated to 65° C. one-half hour and again tested. If inactive at this point various materials were added for reactivation. Rabbit serum and milk were used undiluted, since no hemolyses of rabbit corpuscles are produced by them. The fowl and swine sera were added only in a dilution of 1:10, quantities greater than this causing a solution of rabbit blood. The following table gives a synopsis of one particular series of reactions:

TABLE 7.

ATTEMPTED REACTIVATION FOR RABBIT BLOOD.

Extract IV (65° C. ½ Hr.)	5 per cent Rabbit Blood	Complementai Substances	Lysis
0.5 c.c.	1 c.c.	1.0 c.c. NaCl 1 per cent	None
0.5	1	1.0 rabbit serum	“
0.5	1	0.1 fowl serum	“
0.5	1	0.3 swine serum	“
0.5	1	0.5 cow's milk	“

From these experiments it may be seen that the substances which are capable of reactivating inactivated serum hemolysins, such as native and foreign sera, are unable to restore to the heated *Amanita phalloides* its hemolytic properties. A large number of different combinations of erythrocytes with different blood sera were made, but in no case did any hemolytic activity manifest itself with the extracts heated to 65° C.

Amanita phalloides, therefore, differs in this respect from the serum hemolysins, but is quite similar to the bacterial hemolysins.

Reactions with resistant erythrocytes.—It has already been pointed out that certain extracts of *Amanita phalloides* exert little or no action upon certain varieties of blood, notably that of beef or sheep. Thus Extract IV, whose hemolytic strength for guinea-pig corpuscles is 0.001 c.c. has practically no action upon sheep blood as may be seen from the following table:

TABLE 8.

REACTION WITH SHEEP BLOOD.

Extract IV + 5% Blood 1.0 c.c.	Lysis
Sheep blood (washed) + extract 1.00 c.c.	None
“ “ “ + “ 0.5	“
“ “ “ + “ 0.1	“
“ “ “ + “ 0.05	“
“ “ “ + “ 0.01	“
“ “ “ + NaCl Sol. only (control) . . .	“
Guinea-pig blood + extract 0.001 c.c	Complete
“ “ “ + NaCl sol. only	None

The question comes up whether various sera can furnish the ingredients necessary to bring about complete solution of resistant bloods with extracts of *Amanita phalloides.* A number of reactions were carried out to demonstrate this point, using beef and sheep blood to which extracts of *Amanita phalloides*, active for other corpuscles, were added in large amounts, together with appropriate quantities of sera. These sera were first tested upon the corpuscles in question to determine the amounts which could be employed with impunity. In the following table, which represents the reactions with sheep blood, fowl, rabbit, and guinea-pig sera were added in 0.05 c.c. quantities only.

TABLE 9.

ATTEMPTED ACTIVATION OF *Amanita phalloides* FOR SHEEP BLOOD.

Extract IV	5 per cent Sheep Blood	Activating Material	Lysis
0.1 c.c.	1 c.c.	1.00 c.c. NaCl 1 per cent	None
0.1	1	1.00 sheep serum	"
0.1	1	0.05 fowl serum	"
0.1	1	0.05 rabbit serum	"
0.1	1	0.05 guinea-pig serum	"

A number of similar combinations of *Amanita phalloides* extract with other varieties of resistant bloods and a large assortment of native and foreign sera were made, but in no case did hemolysis result. The substances necessary for the solution of blood corpuscles resistant to extracts of this fungus are not furnished by blood serum.

Reactions with heated sera.—It has been shown by Calmette[32] that certain sera heated to 62.5° C. liberate substances which may act as complements for hemolytic amboceptors.

This reaction has been explained by Kyes as due to the liberation of lecithin, but in view of the possibility that other materials than lecithin might be present in heated serum of a complemental nature it was determined to employ the serum primarily. Beef and swine sera were heated to 65° C. and to 80° C. for thirty minutes, to prevent coagulation the serum being diluted with salt solution. Appropriate quantities were added to mixtures of phalloides extract and resistant corpuscles of beef, sheep, and swine, but in no case was lysis produced. The same sera, when added to inactivated extracts and susceptible erythrocytes of rabbits and guinea-pigs, was without effect upon them. The heating of serum, therefore, does not liberate complement for the hemolysin of *Amanita phalloides.*

Reactions with endocomplement.—The valuable work by Kyes[30] in demonstrating that cobra venom can be made to dissolve resistant blood corpuscles by the addition of extracts made from susceptible corpuscles, the so-called "endocomplement," and in later proving that lecithin is the ingredient of the susceptible corpuscles which plays the part of a complement, has opened up an entirely new field in the study of hemolytic reagents. Kyes's work has been followed by a number of important observations upon other hemolytic substances, notably that of Sachs,[33] who has proved that the laking of blood by sublimate is hastened and increased by lecithin, and that of Pascucci,[34] who has demonstrated that ricin, which outside the body merely agglutinates the blood corpuscles, will dissolve them on the addition of lecithin. It is therefore necessary to determine whether endocomplement made from blood corpuscles susceptible to *Amanita phalloides* can have any influence in activating extracts of this fungus for relatively resistant corpuscles or in reactivating these extracts rendered inactive by heat. For this purpose "endocomplement" was prepared from susceptible corpuscles, rabbit and guinea-pig, by washing these corpuscles carefully, laking them with distilled water, and then adding concentrated salt solution in sufficient quantity to bring the solution up to the requisite tonicity. In a number of reactions this endocomplement was employed for activation and for reactivation, but in all cases the results were negative, as can be seen below.

TABLE 11.
REACTIONS WITH ENDOCOMPLEMENT.
CONTROL WITH *Amanita phalloides*.

EXTRACT K	LYSIS				
	5 per cent Rabbit Blood 1.0 c.c.	5 per cent Beef Blood 1.0 c.c.	5 per cent Beef Blood 1.0 c.c. + Rabbit Endocomplement 1.0 c.c.	5 per cent Sheep Blood 1.0 c.c.	5 per cent Sheep Blood 1.0 c.c. + Rabbit Endocomplement 1.0 c.c.
1.00 c.c.	Complete	None	None	....	....
0.25	"	"	"	None	None
0.10	"	"	"	"	"
NaCl sol. only (control)	None	"	"	"	"

Endocomplement was also employed in a number of other ways for activating *Amanita phalloides* for resistant corpuscles and for reactivating extracts of the fungus heated to 65° C.; in all cases the extracts of the susceptible corpuscles had no influence in determining the solution of blood corpuscles.

Reactions with lecithin.—Lecithin obtained from Merck, dissolved in methyl alcohol, in a 1 per cent solution, then diluted with physiological salt solution, was tested to determine whether it enters into combination with *Amanita phalloides*. These experiments were conducted mainly with beef, sheep, and swine blood, although rabbit and guinea-pig corpuscles were also tested with inactivated *Amanita phalloides* and lecithin. A considerable number of reactions were carried out, in all of which the results were negative. It is necessary, therefore, to give only one or two examples, which are represented in the following table:

TABLE 12.

ACTIVATION WITH LECITHIN.

CONTROL.

Amanita extract was first tested upon 5 per cent rabbit-blood suspension and found to be active n a dilution of 1:100. The undiluted material was inactive for beef blood. Various dilutions of lecithin dissolved in methyl alcohol (1 per cent solution) were tested upon beef blood and found to be inactive in 0.003 c.c. quantities.

Lecithin was now employed in 0.003 c.c. quantities for the activation of *Amanita phalloides* for beef blood.

Extract IV	5 per cent Beef Blood (Washed)	Lecithin, 1 per cent	Lysis
1.0 c.c.	1 c.c.	0.003 c.c.	None
0.1	1	0.003	"
0.05	1	0.003	"
0.01	1	0.003	"
NaCl sol. only (Control)	1	0.003	"

Here it may be seen that lecithin has no action in causing a solution of resistant corpuscles by means of inactive extracts of *Amanita phalloides*. The ingredients of the mixtures were varied, using different quantities of *Amanita phalloides* and various amounts of lecithin, as well as of other fairly resistant corpuscles; but it could not be determined that lecithin exerted any influence in the reactions. Lecithin was also employed for reactivating the heated extracts, but the results were negative.

TABLE 13.

REACTIVATION OF INACTIVATED *Amanita phalloides* WITH LECITHIN.

Extract IV was first tested upon rabbit blood and then inactivated at 65° C. Lecithin was tested upon rabbit corpuscles and found to give a solution of them in quantities greater than 0.003 c.c. This amount of lecithin was therefore employed for reactivation.

Extract IV, 65° C. Half Hour	5 per cent Rabbit Blood (Washed)	Lecithin, 1 per cent	Lysis
1.0 c.c.	1 c.c.	0.003 c.c.	None
0.1	1	0.003	"
0.05	1	0.003	"
0.01	1	0.003	"
NaCl sol. only (Control)	1	0.003	"

COMBINATIONS OF RESISTANT CORPUSCLES WITH *AMANITA PHALLOIDES.*

The question arises whether the failure of certain resistant corpuscles like those of beef, sheep, or swine to dissolve, when combined with certain extracts of *Amanita phalloides* which act vigorously upon other erythrocytes, is due to the lack of certain ingredients in the corpuscles themselves, necessary for hemolysis,or is the result of the inability of these resistant corpuscles to unite with the hemolytic principles. Such a question, from its very nature, is not capable of exact answer. It may be answered approximately by determining whether the extracts of *Amanita phalloides* lose in hemolytic strength for susceptible corpuscles if treated with the more resistant erythrocytes. Sheep corpuscles were combined with extracts of *Amanita phalloides*, which had a definite strength for guinea-pig blood, and after the lapse of sufficient time for hemolytic reaction the corpuscles were removed by centrifugation, and the supernatant fluid again tested for hemolytic activity for guinea-pig blood. These reactions are given in the following table:

TABLE 14.

ABSORPTIVE POWER OF SHEEP BLOOD.

Amanita phalloides extract was first tested upon guinea-pig corpuscles (washed) and found to give a complete solution in 0.003 c.c. quantities. Appropriate quantities of the extract were now combined with sheep blood which is not dissolved.

Extract VI	5 per cent Sheep Blood (Washed)	Lysis
1.0 c.c.	1 c.c.	None
0.8	1	"
0.5	1	"
0.3	1	"
0.1	1	"
NaCl sol. only (Control)	1	"

These tubes were now centrifugalized and the supernatant fluid tested for hemolytic strength for guinea-pig corpuscles. The strength for these corpuscles is unaltered.

Extract VI (from Sheep Blood)	5 per cent Guinea-Pig Blood (Washed)	Lysis
0.25 c.c.	1 c.c.	Complete
0.10	1	"
0.01	1	"
0.008	1	"
0.005	1	"
0.003	1	"
0.001	1	Partial
NaCl sol. only (Control)	1	None

Therefore resistant corpuscles can remove from extracts of *Amanita phalloides* little or none of the hemolytic material which is capable of acting upon susceptible blood.

In the same way extracts of *Amanita phalloides* can be combined with the stroma of resistant corpuscles with practically no change in their hemolytic strength for susceptible erythrocytes.

Thus 2 c.c. of Extract V which gives a complete solution of rabbit corpuscles in 0.003 c.c. quantities was combined with the stroma from 20 c.c. washed beef blood. After 18 hours the supernatant fluid was tested for hemolytic strength for rabbit blood.

TABLE 15.

ABSORPTIVE POWER OF STROMA.

Fluid from Stroma	5 per cent Rabbit Blood	Lysis
0.10 c.c.	1 c.c.	Complete
0.05	1	"
0.01	1	"
0.008	1	"
0.005	1	"
0.003	1	"
0.002	1	None
0.001	1	"
Control 1 c.c. ..... NaCl 1%	1	"

It is evident that the original strength for rabbit blood 0.003 c.c. is maintained in the supernatant fluid.

From these experiments it may be seen that the failure of the resistant corpuscles to dissolve is in all probability due to the failure of the corpuscles to unite with hemolytic ingredients of the extracts, unless another possibility be suggested, that there are multiple hemolysins present in *Amanita phalloides* each acting upon a special variety or group of erythrocytes. This latter question has not been definitely settled. It has been found by experimentation with more concentrated extracts of the fungus, and a greater variety of beef, sheep, and swine bloods, that these corpuscles exhibit great differences in susceptibility at different times, especially if not freed from all traces of serum. Eventually extracts of *Amanita phalloides* were obtained which completely dissolved all varieties of corpuscles, but not, however, in great dilution.

These reactions can be seen in the following table:

TABLE 16.

SYNOPSIS EXTRACT V.

Extract V Prepared from about 50 Grams *Amanita phalloides* and 200 c.c. Distilled Water.

STRENGTH FOR VARIOUS ERYTHROCYTES.

Guinea-pig	0.002 c.c.
Rabbit	0.003
Beef	0.5
Sheep	1.0

We have thus been able to confirm Kobert's observations upon the solvent action exerted upon beef blood. We have never been able to obtain a solution of these corpuscles in such a great dilution as did Kobert, but he employed relatively enormous quantities of the fungus, 1,000 grams, and evaporated the extracts to dryness. Our own purified hemolysins evaporated to dryness have not been tested upon beef blood.

REACTIONS WITH LYMPHOCYTES.

The great susceptibility of red blood corpuscles to extracts of *Amanita phalloides* suggested the possibility of a definite leucolytic action. To test this, lymphocytes were employed, the mesenteric lymphatic glands from rabbits being emulsified with salt solution. Appropriate quantities of this emulsion were combined with extracts of *Amanita phalloides* in various ways but without effect.

1 c.c. extract + 1 c.c. lymphocyte emulsion	No lysis
1 " heated to 65° C. + 1 c.c. lymphocyte emulsion	" "
1 " " " 80° C. + 1 " "	" "
Control 1 c.c. NaCl 1 per cent + 1 " "	" "

There is, therefore, no definite solvent action upon lymphocytes outside the animal body. This experiment does not of course indicate that there may not be definite necrosis of the cells by the prolonged action of *Amanita phalloides* in the tissues.

NATURAL ANTIHEMOLYSINS.

In view of the great desirability of obtaining remedies which can be employed in the immediate treatment of poisoning by *Amanita phalloides*, a number of experiments were undertaken to determine whether any substances exert an antagonistic or an antihemolytic action upon extracts of this fungus. In these experiments native and foreign blood sera, raw and heated, raw and boiled milk and lecithin, were utilized. The extracts were first tested upon such susceptible corpuscles as those from guinea-pigs and rabbits, which were dissolved in 0.005 c.c. quantities. Guinea-pig blood also was dissolved in 0.001 c.c. quantities and rabbit blood partially. Rabbit and guinea-pig sera were added undiluted for their homologous erythrocytes. Raw and boiled milk were employed in any desirable quantity since no hemolysins are present in this substance, at least for the corpuscles in question. Lecithin was used only in 0.003 c.c. quantities. The following table gives a synopsis of the results obtained:

TABLE 17.

NATURAL ANTIHEMOLYSINS.

Extract IV	5 per cent Blood (Washed) 1 c.c.	Antilysins	Lysis
0.005 c.c.	Guinea-pig blood		Complete
0.001	" " "		"
0.005	" " "	1.000 c.c. guinea-pig serum	"
0.001	" " "	1.000 " " "	None
0.005	" " "	1.000 milk	"
0.001	" " "	1.000 "	"
0.005	" " "	0.003 lecithin	Complete
0.001	" " "	0.003 "	"
0.005	Rabbit blood		Complete
0.001	" "		Trace
0.005	" "	0.5 c.c. raw milk	None
0.001	" "	0.5 " "	"
0.001	" "	1 boiled milk	Partial
0.005	" "	1 " "	None
0.001	" "	1 " "	"
0.005	" "	1 rabbit serum	"
0.001	" "	1 " "	"
0.005	" "	1 " " heated to 65° C.	"
0.001	" "	1 " " " 65° C.	"

The influence of other sera, fowl, sheep, beef, and swine, upon the hemolytic activities of *Amanita phalloides* was also tested with rabbit and guinea-pig corpuscles. In all cases the foreign and native serum, exhibited a definite antihemolytic action. The strongest of all substances in this respect proved to be milk, both raw and boiled; and milk may be regarded as a mild natural antidote for *Amanita phalloides.*

REACTIONS WITH CHOLESTERIN.

The discovery of Ransom,[35] working in Meyer's laboratory, that the constituent of the corpuscular stroma which is attacked by saponin, and the ingredient of the blood serum which neutralizes it, is cholesterin, rendered it imperative to determine whether the antihemolytic action exerted upon extracts of *Amanita phalloides* by blood serum and milk can be due to the same substance.

To demonstrate this point, cholesterin was dissolved in ether, in a 1 per cent solution of which 1 c.c. was added to 2 c.c. of phalloides extract. As a control 1 c.c. of ether was added to another tube of the extract and both preparations were placed in the theremostat for three hours. At the end of this time the ether had evaporated and cholesterin crystals had begun to deposit in the cholesterin-phalloides mixture. The two mixtures were now tested for their hemolytic strength upon rabbit corpuscles, at the same time the crude phalloides extract being tested by way of comparison. The results are given below:

TABLE 19.

ACTION OF CHOLESTERIN UPON

HEMOLYSIS PRODUCED BY *Amanita phalloides* IN 5 PER CENT RABBIT BLOOD.

Quantities	Cholesterin Mixture	Other Mixtures	Crude Material
1.0 c.c	Complete	Complete	Complete
0.1	"	"	"
0.05	"	"	"
0.01	"	"	"
0.0075	None	None	None
0.005	"	"	"
0.001	"	"	"

CONTROL.—1 c.c. NaCl 1 per cent+1 c.c. 5 per cent rabbit blood=None.

Hemolysis is produced in rabbit blood by the various mixtures in exactly the same quantity, and cholesterin cannot be considered the ingredient of the serum which exerts this antagonistic action upon *Amanita phalloides*. This action is in all probability due to the albuminous constituents of serum and milk.

SUMMARY OF HEMOLYTIC REACTIONS.

From these experiments it may be seen that extracts of *Amanita phalloides* contain a hemolytic principle which has been isolated by Kobert to which he gave the name "phallin." This substance dissolves all varieties of erythrocytes which exhibit great differences in susceptibility. It enters into direct combination with the red blood cells, dissolving them without the intermediation of serum. It is inactivated at a temperature of 65° C. acting for one-half hour; this inactivation is apparently a destruction, since all substances capable of reactivating serum hemolysins, including serum, corpuscular extracts, and lecithin, fail to restore to this heated material its hemolytic properties. Its activity is inhibited by blood sera and by raw and boiled milk, the latter of which may be looked upon as a natural antidote of mild strength. In virtue of these properties phallin should be provisionally classed with the bacterial hemolysins.

4. ACTION OF *AMANITA PHALLOIDES* UPON ANIMALS.

If we inoculate small animals such as rabbits or guinea-pigs with minute quantities of extracts made from *Amanita phalloides* it is found that this substance is possessed of extreme toxicity. Thus in an extract prepared from 8 grams of dried *Amanita phalloides* and 100 c.c. of distilled water, 1 c.c. will kill a rabbit of 2,000 gram weight in 48 hours, and 0.5 c.c. will kill a guinea-pig of 500 gram weight in the same time. Within a few hours after the inoculation the animals show signs of poisoning, slinking to a corner of the cage with ruffled fur and refusing to eat. They rapidly lose weight and strength, when placed on their back or sides being unable to get to their feet. After a period of time varying from 24 hours to three days they develop great difficulty in breathing, the respirations being much diminished in frequency and increased in depth. The respirations gradually cease, while the heart continues to beat forcibly for some seconds after the respirations are no longer visible. If the chest wall be opened immediately, the heart may be found to be still beating.

With small, feeble guinea-pigs death may occur within a few hours after subcutaneous inoculation, but with normally healthy guinea-pigs and rabbits the period of illness lasts usually one to six days.

Small doses induce a chronic intoxication, especially in rabbits, which die at the end of three, four, or even six weeks. Guinea-pigs, if they survive 12 to 15 days, practically never die of the late changes. Convulsions or convulsive movements do not occur ordinarily, although convulsions may be seen as a rare terminal event. Salivation and gastro-intestinal symptoms are never present and phalloides poisoning may thus be clearly differentiated from muscarin poisoning.*

Postmortem changes.—The lesions following subcutaneous inoculation are definite and characteristic. There is seldom postmortem rigidity. At the site of inoculation there is a most extensive subcutaneous edema, with numerous small areas of hemorrhage scattered about in the edematous tissue and in the adjacent muscles. The subcutaneous lymphatic glands are swollen and hemorrhagic. The serous membranes are free from excess of fluid, but there may be

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**Amanita phalloides* has no action upon the cornea, such as that shown by ricin.

hemorrhages in the mesentery and occasionally in the pleura. The adrenals are always hemorrhagic, and there may be hemorrhages in the mesenteric lymphatic glands. The liver and kidneys occasionally show hemorrhagic areas on their surfaces, and there are always minute areas of hemorrhage in their interior. The bladder is filled with urine which may be blood stained. The spleen is enlarged and soft. The lungs may show small hemorrhages, which may also rarely be present on the pericardial surface of the heart. This organ is always greatly dilated, the contained blood being fluid or rarely clotted. The blood in the larger vessels is occasionally fluid, and in the acute cases is much darker in color than is the normal blood. The stomach contents are frequently blood stained, and minute ulcers are found in the submucosa with small hemorrhages at their bases. Both brain and spinal cord also show definite areas of hemorrhage.

With small doses, when the animals die of chronic intoxication, the lymphatic glands and adrenals are usually hemorrhagic, but the most marked changes are the extreme fatty degeneration and the necrosis of the liver and other organs.

Microscopic examination.—Microscopically the changes seen are similar to those already described. The evidence of hemorrhage in lymphatic glands, in liver, spleen, and kidneys is well marked. There are countless red blood corpuscles scattered through the tissues, and occasionally degeneration of the endothelial lining of the blood vessels may be discerned. An extensive deposition of blood pigment has taken place in the spleen. The muscle fibers of the heart show hyaline degeneration, and minute areas of necrosis with small-celled infiltration are occasionally found. There is an extensive deposition of fat in various regions, but most marked in the liver, and necrotic cells may be found in nearly all the organs.

The following protocol is a fair example of the effects of subcutaneous inoculation:

Guinea-pig weighing 715 grams inoculated January 20, 1905, with 1 c.c. of an extract of *Amanita phalloides* made from 8 grams dried material and 100 c.c. distilled water. The following day the animal was found in the corner of the cage with ruffled fur and refusing its food. It gradually developed great weakness. When placed on its side it got to its feet only with difficulty. There was no salivation, no convulsive movements, no diarrhea. The respirations were much lessened in frequency and increased in depth. Gradually increasing dyspnea was followed by death at 4 P. M., January 24, four days after inoculation.

Autopsy was held at once. An enormous gelatinous edema was present over the entire anterior abdominal wall with small hemorrnages present in this edematous tissue. Subcutaneous lymphatic glands swollen and hemorrhagic. Peritoneal cavity free from exudate. Vessels of mesentery injected. Spleen congested, dark, and friable. Liver surface shows several small hemorrhages and several opaque areas of fatty degeneration. Adrenals hemorrhagic; kidneys apparently normal; bladder filled with blood-stained urine; pleural cavity free from fluid; lungs show a few small areas of hemorrhage; heart much dilated; blood fluid, but clots on exposure to the air; brain and spinal cord also show hemorrhagic areas.

Cultures from heart, liver, and peritoneal cavity were sterile.

ESTIMATION OF MINIMUM FATAL DOSES.

By inoculating a considerable series of animals with graduated doses of phalloides extracts it is possible to determine, within narrow limits, the quantity which will kill animals of a definite weight. Two different limits of toxicity may be made out, the smallest quantity which will kill an animal acutely and the smallest quantity which will kill an animal by chronic intoxication. Then by inoculating another series of animals with a quantity somewhat in excess of the dosage already estimated, that amount of *Amanita phalloides* which will always kill animals of a definite weight in a definite time may be determined. This quantity of any extract may then be considered the M. L. D. of that extract. By comparing the minimum fatal dose with the hemolytic strength, we obtain a ratio of toxicity to hemolysis which gives us a rough guide for estimating the toxicity of new solutions. With extracts freshly made from the dried fungus, this ratio of toxicity to hemolysis is fairly constant, and by making two or three control inoculations to discover whether the poisons have degenerated we are in possession of sufficient data in regard to any particular extract to guide us in experimental work. A considerable number of different extracts of the fungus were compared for hemolytic strength and for toxicity upon both rabbits and guinea-pigs, and it was learned that not only is the quantity of hemolysin present a fairly accurate guide to the toxicity, but that both hemolytic activity and toxicity suffer little impairment from the passage of time.

The toxicity of Extract I, whose hemolytic reactions have already been carefully considered, is given in the following table:

TABLE 20.

TOXICITY OF EXTRACT I FOR RABBITS.

Weight in Grams	Dosage	Interval before Death
1,220	2.0 c.c.	48 hours
850	1.5	24 "
870	0.5	48 "
2,000	0.3	14 days
1,850	0.2	6 "

Minimum fatal dose for rabbits 2,000 gr. . . . . 0.3 c.c.
" " " " " 1,500 " 0.2
Dose sufficient to cause acute death in rabbit of 1,000 grams . . 0.5
Hemolytic strength for rabbit blood 0.05

TOXICITY OF EXTRACT I FOR GUINEA-PIGS.

Weight in Grams	Dosage	Result
552	2.0 c.c.	Death in 3 days
715	1.0	" " 4 "
455	1.5	" " 1 day
250	0.2	" " 1 "
240	0.1	" " 1 "
437	0.1	Recovery
365	0.075	"
427	0.05	"

Minimum fatal dose for guinea-pig 500 grams . . . . 0.5 c.c.
" " " " " 250 " 0.2
Hemolysis for guinea-pig blood 0.005

The strength of other extracts was also tested upon animals. Thus 0.34 c.c. of Extract IV made from 15 grams dried *Amanita phalloides* and 200 c.c. distilled water, whose hemolytic strength for rabbit blood was 0.002 c.c., killed a rabbit of 847 grams in less than 24 hours, with the usual appearances of gelatinous edema, hemorrhages, and degenerations.

Again the toxicity of Extract V prepared from 30 grams dried *Amanita phalloides*, and 200 c.c. distilled water, whose hemolytic strength for guinea-pig blood was 0.002 c.c., was tested upon guinea-pigs with the following result:

Weight in Grams	Dosage	Result
579	1.0 c.c.	Death in 3 days
499	0.5	" " 24 hours
429	0.25	" " 24 "

Here it may be seen that the toxicity of Extract V is somewhat greater than that of Extract I, 0.25 c.c. sufficing to kill a guinea-pig of 427 gram weight in 24 hours.

5. IMMUNIZATION OF ANIMALS.

If we treat animals with gradually increasing doses of *Amanita phalloides* it is possible to accustom them to the material, provided the initial dose be much under that capable of causing a chronic intoxication. This immunization must be carried out over a period of many weeks, as the rapid repetition of the doses, and the increase in their amount causes a high mortality. By treating a number of rabbits with various quantities of *Amanita phalloides* extracts, at various intervals of time eventually the proper method of successfully immunizing them was worked out, and a number of rabbits were immunized to the point where they would withstand the inoculation of rapidly-fatal doses, and several multiples of fatal doses.

The following table gives a synopsis of the treatment of one of the successfully immunized rabbits:

TABLE 21.

IMMUNE RABBIT A, WEIGHT 2,020 GRAMS.

Immunized to Extract IV whose Hemolytic Strength for Rabbit Blood is 0.002 c.c.

Date of Inoculation	Quantity given	
February 9	0.002 c.c.	
“ 11	0.004	
“ 13	0.008	
“ 15	0.010	
“ 20	0.032	
“ 25	0.064	
March 6	0.14	
“ 11	0.25	1 fatal dose
“ 17	0.4	
“ 24	0.6	2 fatal doses
“ 31	0.8	
April 7	1.0	3–4 fatal doses

Bled April 14, 20 c.c.

ANTIHEMOLYTIC PROPERTIES OF IMMUNE SERA.

At this point the animals were bled and their sera subjected to a careful study upon blood corpuscles and upon animals. Primarily such blood sera is *antihemolytic.* It completely inhibits the solution of blood corpuscles by *Amanita phalloides* and in considerable dilutions. If we estimate the smallest quantity of any extract which will completely dissolve 1 c.c. of a 5 per cent suspension of guinea-pig or rabbit blood corpuscles, the immune serum from Rabbit A completely arrests this solution of corpuscles in a dilution of 1:1,000. Thus the serum from Rabbit A was tested for antihemolytic properties on April 15 with the results shown in the following table:

TABLE 22.

ANTIHEMOLYTIC STRENGTH OF SERUM RABBIT A.

CONTROL *Amanita phalloides* EXTRACT.

Extract V+5 per cent Rabbit Corpuscles 1.0 c.c. +Immune Serum				Lysis
Extract V	0.005 c.c.	+ 1.0 c.c. NaCl	1%	Complete
"	0.003	+ 1.0 "	1	"
"	0.002	+ 1.0 "	1	Partial
"	0.001	+ 1.0 "	1	Trace
"	0.005	+ normal serum	1.0 c.c.	Complete
"	0.005	+ immune "	1.0	None
"	0.005	+ " "	0.01	"
"	0.005	+ " "	0.0075	Faint trace
"	0.005	+ " "	0.001	" "
"	0.005	+ " "	0.0001	Partial
"	0.003	+ normal "	0.1	Complete
"	0.003	+ immune "	0.1	None
"	0.003	+ " "	0.01	"
"	0.003	+ " "	0.0075	"
"	0.003	+ " "	0.001	"
"	0.003	+ " "	0.0001	Complete
NaCl sol. only (control)				None

The serum from Rabbit A therefore has an antihemolytic strength for rabbit corpuscles of 1:1000.

The same serum was tested upon guinea-pig corpuscles with similar results.

TABLE 23.

ANTIHEMOLYTIC STRENGTH FOR GUINEA-PIG BLOOD.

CONTROL *Amanita phalloides* EXTRACT.

Extract V+5 per cent Guinea-Pig Corpuscles 1.0 c.c. +Immune Serum				Lysis
Extract V	0.005 c.c.	+ 1.0 c.c. NaCl sol.	1%	Complete
"	0.003	+ 1.0 " "	1	"
"	0.002	+ 1.0 " "	1	"
"	0.001	+ 1.0 " "	1	Partial
"	0.002	+ normal serum	1.0 c.c.	Complete
"	0.002	+ immune "	1.0	None
"	0.002	+ " "	0.1	"
"	0.002	+ " "	0.01	"
"	0.002	+ " "	0.0075	"
"	0.002	+ " "	0.001	"
"	0.002	+ " "	0.0001	Partial
NaCl sol. only (control)				None

Therefore, the serum of Rabbit A has an antihemolytic action upon guinea-pig corpuscles in a dilution of 1:1000.

ANTITOXIC PROPERTIES OF IMMUNE SERA.

The blood serum of these immune animals besides possessing antihemolytic properties is definitely *antitoxic.* If doses of *Amanita phalloides* much in excess of minimum fatal doses be mixed with appropriate amounts of the immune serum, they can then be administered to susceptible animals without deleterious effects. Such antitoxic properties are shown in the following table:

TABLE 24.

ANTITOXIC ACTION OF IMMUNE SERUM A.

CONTROL.—Rabbit weighing 2,000 grams inoculated with 1 c.c. of Extract V. Death in 48 hours with typical lesions.

Serum tested upon seven rabbits, all well under 2,000 grams weight.

Dosage	Amount Immune Serum	Result
1 c.c.	3.0 c.c.	No edema site of inoculation
1	2.0	" " " " "
1	1.5	" " " " "
1	1.0	Edema site of inoculation
1	0.5	" " " "
1	0.25	" " " "
1	0.125	" " " "

All of these animals were watched for a period of 10 weeks, during which the edema at the site of inoculation disappeared and the animals remained free from any signs of chronic intoxication. Quantities of antiserum from Rabbit A above 1 c.c. completely neutralize three to four times a fatal dose of *Amanita phalloides* for rabbits of 2,000 grams, and quantities less than 1 c.c. save the animals from death. The same serum possesses antitoxic properties for guinea-pigs.

A number of rabbits were successfully immunized to the same point as was Rabbit A, but to be absolutely certain that one is dealing with a definite antitoxin, it is necessary to produce a higher degree of resistance and to obtain a serum which will neutralize larger multiples of fatal doses. With small animals it is not possible to produce antitoxic sera of higher potency, but it seemed desirable to obtain one in which 1 c.c. would neutralize at least 10 times a fatal dose for a susceptible animal, the standard originally set up by Ehrlich upon von Behring's work, as a *normal serum.* Many difficulties were encountered when the attempt was made to make rabbits resistant to large doses of *Amanita phalloides*, as these animals were completely overwhelmed by the excessive doses and frequently died acutely. Eventually by beginning the immunization with subcutaneous inoculations and following this intraperitoneal inoculation of the larger quantities several animals were successfully handled.

The details of the immunization of one of these animals are given in the following table:

TABLE 25.

IMMUNE RABBIT C.

IMMUNIZED TO EXTRACT IV. WEIGHT 2,000 GRAMS.

Date	Dosage	
February 9	0.002 c.c.	
“ 14	0.004	
“ 20	0.008	
“ 25	0.016	
March 5	0.032	
“ 9	0.064	
“ 14	0.14	
“ 18	0.25	fatal dose
“ 24	0.4	
“ 31	0.6	2 fatal doses
April 7	0.8	
“ 14	1.0	3–4 fatal doses
May 1	1.0	3–4 “ “
May 8, bled 45 c.c.		
May 18	0.75 c.c.	3 fatal doses
“ 26	1.0	3–4 “ “
June 6	1.25	4 “ “
“ 19	1.5	4–5 “ “
“ 28	2.0	6–7 “ “
Bled July 6, 1905.		

The serum from Rabbit C was tested May 8 after it had been successfully immunized to withstand acutely fatal doses. At this point it had an antihemolytic strength of 1:1,000. Its antitoxic strength was tested only with an excess of *Amanita phalloides* as is shown in the following table:

TABLE 26.

CONTROL RABBIT INOCULATED WITH 2 C.C. EXTRACT V, REPRESENTING SIX TO SEVEN TIMES A FATAL DOSE.

DEATH 18 HOURS.

I. RABBIT.—Inoculated May 9, 2 c.c. Extract V and 3 c.c. antiserum. Developed some edema at site of inoculation. This disappeared and the animal remained perfectly well for 10 weeks.

II. RABBIT.—Inoculated May 9, 2 c.c. Extract V and 2 c.c. antiserum. Death in 24 hours.

III. RABBIT.—Inoculated May 9, 1 c.c. Extract V and 1.2 c.c. antiserum. This animal developed edema at the site of inoculation, but this disappeared completely and the animal remained well.

From this series of animals it may be seen that at the time when the serum from Rabbit C had an antihemolytic strength of 1:1,000 it was possessed of definite antitoxic properties. Thus 2 c.c. of Extract V, representing six to seven times a fatal dose for rabbits of 2,000 grams, is completely neutralized by 3 c.c. of the antiserum, while half that quantity of the extract, representing three to four times a fatal dose, requires a little more than 1 c.c. serum for neutralization. Mixtures of phalloides extract and serum in which the proportion of serum is below this ratio are not neutralized.

At this point, although definite multiples of fatal doses had been neutralized, it was determined to push the animal still further, and the inoculations were continued over another period of two months. Considerable intervals of time were allowed to elapse between the doses, which were given intraperitoneally to avoid the profound local necrosis produced by the subcutaneous inoculation of large quantities of phalloides extract.

This particular animal stood the injections well, finally receiving 2 c.c. of Extract V, representing six to seven times a fatal dose for a rabbit of 2,000 grams. All told the animal received 11 c.c. of this solution, representing about 33 times a fatal dose for a rabbit of its weight. Its serum was finally again withdrawn after the last inoculation and tested for antihemolytic and antitoxic strength. At this time it would prevent hemolysis by *Amanita phalloides* in a dilution of 1:10,000 and 1 c.c. of the serum would neutralize 2 c.c. of Extract V, as can be seen in Table 27:

TABLE 27.

I. RABBIT.—2 c.c. Extract V—2 c.c. Antiserum C—Recovery
II. RABBIT.—2 c.c. Extract V—1 c.c. Antiserum C—Recovery
III. RABBIT.—2 c.c. Extract V—0.5 c.c. Antiserum C—Death
IV. RABBIT.—2 c.c. Extract V—0.25 c.c. Antiserum C—Recovery

The average weight of these rabbits was 1,500 grams. From this table it may be seen that 1 c.c. of the serum of Rabbit C, which has an antihemolytic strength of 1:10,000, possesses also an antitoxic strength of such an amount that 1 c.c. will neutralize six to seven times a fatal dose for a rabbit of 2,000 grams and 10 times a fatal dose for a rabbit of 1,500 grams. Such a serum may roughly be compared with an antitoxic serum of low potency in that 1 c.c. neutralizes 10 minimum fatal doses.

CELLULAR IMMUNITY.

The question arises, in the consideration of several problems of immunity, whether the cells of the body acquire any resistance to the action of toxins in immunization, or whether protection from fatal doses is due to the antitoxic properties which the surrounding blood serum possesses. With a hemolytic agent, such as phalloides extract, which acts so vigorously upon the erythrocytes this question is easily settled for these particular cells.

Blood fromRabbit C, which was completely immunized to *Amanita phalloides*, was withdrawn and tested in the usual manner. This blood in 5 per cent suspension was tested without washing and after being washed five times. The results may be seen in the following table:

TABLE 28.
Cellular Immunity.
control *Amanita phalloides* extract.

Extract V +5 per cent Rabbit Blood 1.0 c.c.	Lysis		
	Normal Rabbit Blood	Blood Rabbit C Unwashed	Blood Rabbit C Washed 5 Hours
Extract 0.10 c.c.	Complete	Complete	Complete
" 0.04	"	None	"
" 0.02	"	"	"
" 0.01	"	"	"
" 0.005	Partial	"	Partial
NaCl sol. only (control)	None	"	None

From these observations it is apparent that, whereas the unwashed corpuscles of the immune rabbit exert considerable resistance to hemolysis by *Amanita phalloides*, the corpuscles deprived of their serum and suspended in normal saline solution are susceptible to the same extent as are the normal corpuscles of the same species. The resistance of the corpuscles to solution by the hemolysin is therefore due to the antihemolytic properties of the blood serum in which the corpuscles are suspended, the corpuscles themselves possessing no increased resistance. There is therefore *no cellular immunity* possessed by the erythrocytes of animals highly immunized to *Amanita phalloides*.

6. CONCLUSIONS.

In consideration of the results obtained from the investigations reported in this paper, it may be stated that the poisons of *Amanita* *phalloides*, of which the phallin of Kobert is the hemolytic constituent, are of the nature of toxins in that they act upon the animal body after a definite latent period, produce lesions which are characteristic of bacterial intoxications in general, and produce immunity in susceptible animals by the inoculation of non-lethal doses. Such immunized animals will eventually withstand the inoculation of considerable multiples of fatal doses and their blood serum possesses antihemolytic and antitoxic properties, a serum eventually being obtained in which 1 c.c. will neutralize 10 times a fatal dose of the poison. Whether sera of greater potency can be produced by the treatment of larger animals is a problem for the future to decide, but in favor of the attempt to produce sera which can be used for curative purposes in man may be mentioned the following facts: The number of cases of fungus poisoning is numerous, far more so than is generally recognized, and the vast majority of all deaths from this cause are due to a single species, *Amanita phalloides;* the mortality is high, varying from 60 to 100 per cent; the clinical symptoms are characteristic, the suffering is extreme, and the diagnosis is easily made from the history; the physician is called to the case almost at once, and a period of six to eight days in adults and three to four days in children elapses between the time of poisoning and the fatal outcome, during which curative sera could be administered.

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